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Kinetic study of aggregation of milk protein and/or surfactant-stabilized oil-in-water emulsions by Sedimentation Field-Flow Fractionation

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ABSTRACT

Milk proteins are able to facilitate the formation and stabilization of oil droplets in food emulsions. This study employed Sedimentation Field-Flow Fractionation (SdFFF) to monitor changes in particle size distribution of freshly prepared emulsions with varying weight contributions of sodium caseinate (SC) and whey protein concentrate (WPC). The effect of the addition of Tween 80 (T) on the initial droplet size was also investigated. The results indicated that emulsifying ability follows the order Tween 80 > WPC > SC, with corresponding weight average droplet diameter of 0.319, 0.487 and 0.531 μm respectively, when each of the above emulsifiers was used solely. The stability of sodium caseinate emulsions was studied at 30.5 and 80.0 °C by measuring the particle size distribution for a period of 70 h. Emulsions withstood the temperatures and exhibited an initial increase in particle size distribution caused by heat-induced droplet aggregation, followed by a decrease to approximately the initial droplet size. The rate of droplet aggregation depends on the severity of thermal processing, as revealed by the kinetics of particle aggregation during aging at different temperatures. Comparison of the experimental rate constants found from SdFFF, with those determined theoretically gives invaluable information about the oil droplet stability and the aggregation mechanism. Based on the proposed mechanistic scheme various physicochemical quantities, which are very important in explaining the stability of oil-in-water emulsions, were determined. Finally, the advantages of SdFFF in studying the aggregation of the oil-in-water droplets, in comparison with other methods used for the same purpose, are discussed.

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1. Introduction

Milk proteins, thanks to their amphiphilic nature, are well-known emulsifying agents used in the food industry for the production of various products such as salad dressings, coffee whiteners, and cream liqueurs. Numerous studies, which aim to investigate the adsorption behavior of milk proteins, in binary or more complex mixtures, at the oil interface can be cited [1–4]. In an effort to elucidate the mechanism of competitive adsorption of milk proteins sequential experiments have been conducted, where one protein was introduced following emulsion formation by another protein [5]. Nevertheless, the detailed mechanism resulting to a given interfacial composition, particularly in complex protein mixtures, is not yet fully understood.

In emulsions stabilized with milk proteins, competitive adsorption between the protein fractions occurs at the oil-in-water interface [6]. A kinetically controlled mechanism, according to which the first protein to adsorb at the interface is more likely to remain there during storage or further processing, determines to large extent the dominant protein species at the stabilizing layer of the oil droplet [7,8]. Thus, in emulsions stabilized with mixtures of milk proteins, caseins being structurally hydrophobic and flexible tend to adsorb preferentially at the interface compared to whey proteins, because of their ability to reach faster the destination and fulfill their thermodynamic purpose, which is the lowering of the surface tension [9,10]. Furthermore, milk proteins may compete for the oil droplet surface with another class of adsorbing species, formerly known as small molecule surfactants. Non-ionic surfactants (polar lipids and their derivatives) can effectively displace milk proteins from the emulsion interface depending on the relative surfactant/protein ratios and the processing conditions [8,11].

Field-Flow Fractionation is a technique suitable for separation and characterization of colloidal materials and macromolecules

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Table 1
SdFFF instrumentation and method setup.

Instrument settings	Operating conditions	Milk proteins	Milk proteins/Tween 80
Analysis type	Normal (field programming)		
Initial field strength		800 rpm	1000 rpm
Final field strength	50 rpm		
Equilibration time	15 min		
Time held at initial field strength after equilibration time, t_1	10 min		
Field decay parameter, t_α	−80 min		
Parameter p (Eq. (2))	8		
Channel flow rate		1.5 ml/min	1.0 ml/min
Data rate 7	Points/min		

[12–19]. SdFFF, a sub-technique of Field-Flow Fractionation (FFF), is an established elution based separation technique, the theory of which is well documented [12]. The main principle is based on a coupling between the parabolic velocity distribution through a ribbon like channel and a perpendicular field which compresses suspended particles into layers against one wall of the channel. In SdFFF separation is accomplished by introducing centrifugal field forces on the particles suspended in a carrier liquid and colloidal particles are separated by differences in their buoyant mass. This study employed SdFFF, a technique which has been used in the past for separation and characterization of colloidal particles such as the fat globules of oil-in-water emulsions stabilized with milk proteins [20,21].

A limited number of studies are available today which focus on adsorption phenomena by complex mixtures of milk proteins at the oil interface in which the different protein fractions co-exist during the emulsification process at different ratios [22,23]. The quantitative effect of two commercially available milk-protein products (sodium caseinate and whey protein concentrate), used at different proportions, on the size of the fat globule formed following emulsification was investigated. The effect of the addition of Tween 80, a non-ionic surfactant, on the physicochemical properties of the emulsions was also examined. Finally, emulsion stability studies using sodium caseinate were carried out and particle size measurements were used to study the kinetics of oil droplet aggregation during aging at different temperatures. Rate constants of droplet aggregation of heated sodium caseinate emulsions were calculated and a mechanism which might account for the instability phenomena observed was proposed.

The aim of this work is to show how the experimental findings of a separation method, like the SdFFF can be used with success, in combination with theoretical equations, for the prediction of a mechanism for the aggregation of oil-in-water emulsions. The experimental data in combination with theoretical predictions lead also to the determination of important physicochemical quantities for this process, like rate constants of the elementary steps and stability factors of the aggregates. It is noteworthy that both above attainments are presented, as far as we know, for the first time in the literature proving the novelty of our work, as well as its significance to separation scientists dealing with foods.

2. Materials and methods

2.1. Reagents and chemicals

Potassium hydroxide and sodium azide were purchased from Merck (Germany). Commercially available corn oil was supplied from the local supermarket (Aro, Makro Hellas). Sodium caseinate (from bovine milk) was purchased from Sigma–Aldrich (USA), whey protein concentrate from Solgar (USA) and Tween 80 Biochemica from Applichem (UK). FL-70, a low-foaming, low-alkalinity, phosphate-chromate- and silicate-free mixture of anionic and non-ionic surfactants was supplied from Fisher Scientific (UK).

2.2. Emulsion preparation

Milk protein dispersions were initially prepared by adding corn oil (15%, w/w) and the emulsifier (3% (w/w), protein or surfactant) to triply distilled water and then allow stirring for 1 h at room temperature (∼25 °C) to ensure complete dispersion. Sodium azide was also added (0.1%, w/w) to prevent microbial growth and the pH of the dispersion was adjusted to 7 with KOH (1 M). Ingredients of the emulsion were calculated to give a final weight of 500 g each time, which was the appropriate amount of sample required for homogenization. Samples were processed in a two-stage valve homogenizer (APV-1000, Denmark) working at an operating pressure of 30 MPa. Each sample was passed ten times (recycling) through the homogenizer to ensure complete emulsification. Emulsions were prepared in triplicate and results were averaged.

2.3. Sedimentation Field-Flow Fractionation (SdFFF)

The SdFFF used was the S-101 Particle/Colloid fractionator purchased from Postnova Analytics (Germany). The SdFFF channel was a stainless steel ribbon like channel 89.5 cm long (tip to tip), 2.0 cm wide and 0.0254 cm thick. The void volume (V^0) was 4.45 ml, the injector to channel dead volume was 0.14 ml and the rotor radius of 15.1 cm. The SdFFF system was equipped with an HPLC pump (Model PN 1121 Solvent delivery system, Postnova) and the eluted particles were detected using a UV–vis detector (Model S 3210, Postnova) at the fixed wavelength of 254 nm. The signal was recorded and processed by the computer using in-house FFF analysis software (SPIN 130, Utah, USA) to generate fractograms. Milk samples (20 μl) were injected into a Rheodyne model PN 5100 manual injector. The carrier liquid was triply distilled deionized water containing 0.1% (v/v) FL-70 detergent and 0.02% (w/v) sodium azide as a bacteriocide. The choice of this dispersing medium was based on previous work [24] according to which the surfactant FL-70 stabilizes the oil-in-water emulsions. The channel flow rate was set at 1.5 ml/min for the experimental part which involved emulsion samples containing solely milk proteins and at 1.0 ml/min for the part where the emulsion samples also contained Tween 80. In the latter case the initial field strength was set at 1000 rpm, whereas in all other cases it was 800 rpm. All the samples were analyzed using field programming, described by Eq. (2). The SdFFF operating conditions for milk protein/emulsifier samples are summarized in Table 1.

Following emulsion formation, all samples were sonicated for 5 min (Sonica tuttnauer-2200M, The Netherlands) and diluted 80 times in the carrier liquid before injection into the SdFFF channel to avoid an overloading effect. Densities of the corn oil-in-water droplets were measured, by a simple densitometer, to be

0.9174 g cm^{-3} at 25 °C, 0.9083 g cm^{-3} at 30.5 °C and 0.8740 g cm^{-3} at 80.0 °C.

2.4. Emulsion stability

Two emulsions were prepared, according to the method described in the emulsion preparation section, using sodium caseinate as emulsifier. Emulsion stability has been characterized in terms of changes in the average particle size parameter versus time. Heat-exposure of the emulsions at two temperatures (30.5 and 80.0 °C) was employed to accelerate the sample aging. Samples were placed in plastic test tubes and were heated in an electrically heated oven with a fan mounted in its back wall to provide air circulation (Pye Unicam, Series 104, Cambridge, UK) for a total period of 70 h without stirring. At certain time intervals a small amount of the emulsion was taken for dilution and particle size determination using SdFFF. The temperature inside the oven was followed using a thermometer with a temperature probe located in the oven interior and the timing started when the desired temperature was reached (±0.5 °C). The kinetics of oil droplet aggregation for each temperature was followed by determining the aggregation rate constants for each emulsion from particle size measurements recorded from the stage at which an increase in the weight average diameter, $\bar{d}_w$, values was observed (up to 7 h).

2.5. Optical microscopy

Emulsions were gently agitated in a glass test tube before analysis to ensure that they were homogeneous. A drop of emulsion was placed on a microscope slide then covered with a cover slip. The microstructure of the emulsion was observed using an Olympus CX21 optical microscope (Olympus America Inc.) at 40× magnification. Pictures were taken using a microscope digital imager at 10× magnification (Celestron, USA), installed on a computer.

2.6. Scanning electron microscopy

SEM images were obtained by using a Jeol JSM-5200 Scanning Electron Microscope (SEM) from JEOL (Tokyo, Japan). The oil-in-water droplets were air dried in room temperature and then were sputter-coated with gold before examination [25].

2.7. Statistical analysis

Measurements were carried out in triplicate and data are presented as the mean and standard deviation. The Student *t*-test [26] was used to detect significance of differences among means of particle size distributions ($\bar{d}_w$) when the relative contributions of sodium caseinate and whey protein concentrate in stabilizing the oil-in-water emulsion were compared. Confidence levels were set at 95% ($P < 0.05$). One-way analysis of variance (ANOVA) was used to detect significant differences among means of particle size distributions at 30.5 and 80.0 °C as a function of heating time ($P = 0.05$).

3. Results and discussion

3.1. Contribution of sodium caseinate and WPC to the average droplet size of oil-in-water emulsions

In SdFFF, when the field strength is kept constant, the retention parameter λ is given by the relation [27]:

$$\lambda = \frac{R}{6} = \frac{V^0}{6V_r} = \frac{t^0}{6t_r} = \frac{6kT}{\pi d^3 \Delta\rho G w} \quad (1)$$

where R is the retention ratio, V^0 and V_r are the void and the retention volume, respectively, t^0 and t_r are the void and the retention time, respectively, d is the spherical particle diameter for monodisperse samples (for polydisperse samples the weight average diameter, $\bar{d}_w$, is used), $\Delta\rho$ is the density difference between the particle and the suspending medium, $G = \omega^2 r$ is the centrifugal acceleration, w is the channel thickness, T is the absolute temperature and k is the Boltzmann constant.

In common practice, as in our case, to program a decay of field strength during an analysis so that the separation of a broad distribution of sample components can be accomplished in a reasonable time. The most common field programming for SdFFF is described by the power decay function [27]

$$S = S_0 \left(\frac{t_1 - t_\alpha}{t - t_\alpha} \right)^p \quad (2)$$

where the field strength S is held constant at an initial level S_0 for a pre-decay period t_1 and decreased according to a power function of elapsed time t, as it is described in the experimental section. The second time constant t_α equals to $-8t_1$ [27], as the power p of Eq. (2) is usually set to 8 in order to obtain constant fractionating power.

The retention time for well-retained analytes is then related to field decay parameters as follows [27]:

$$t_r = 9t_1 \left(\frac{t^0}{6t_1\lambda_0} \right)^{1/9} - 8t_1 \quad (3)$$

where λ_0 is the λ value at the initial field strength S_0.

The transformation of the fractogram into a size distribution plot is made by the combination of Eqs. (1) and (3) for elution during field decay and the approximation $t_r = t^0/6\lambda_0$ for the pre-decay period.

In our work, the Nova FFF SF3 Control, FFF Analysis 2.2 software for programmed field conditions, which has been installed in SdFFF S101 system by Postnova Analytics, converted the raw fractograms to the corrected droplet size distribution by removing first the void peak (the end of the void peak was set at 10 min, i.e., for $t > 2t^0$) according to the theory presented previously and using the densities of the corn oil droplets mentioned in the experimental section. The program, which was based on a previous publication [28], removes the size dependence of the detector signal, because it takes into account all the specific information of the particular sample and the operating conditions of the SdFFF system. But, even this correction was not made, the results concerning the kinetics of aggregation should had satisfactory precision, as we are mainly interested in the variation of the size of the oil droplets with the time, and not for the determination of their absolute values. In addition, due to the high polydispersity of the oil droplets, the correction of the average diameters due to the size dependence of the detector signal should be meaningless.

As the oil-in-water droplets are polydisperse (cf. Fig. 3), their weight average diameters were calculated as follows: the data file of the program converting the raw fractogram to particle size distribution contains a great number, depending on the run time and the selected data rate (approximately between 303 and 393), of pairs of x and y values in the corrected fractogram. As the size distribution curve is a mass based distribution, the diameter in the x-axis of the corrected fractogram is a mass based diameter, e.g., a weight diameter, d_{w_i}. The y-axis of the fractogram gives the relative mass, $w_{r_i} = w_i/w_{\text{tot}}$, were w_i is the weight of the n_i droplets having constant diameter d_{w_i} and w_{tot} is the total weight of the eluted sample. Then $w_{r_i} \times dd_{w_i}$ is the weight fraction of the particles having a size comprised between d_{w_i} and $d_{w_i} + dd_{w_i}$, where dd_{w_i} is the increment in d_{w_i} corresponding to increment dV_{r_i} in V_{r_i} at point i along the fractogram. Thus, using the known values of d_{w_i} corresponding to each increment of the x-axis, as well as the

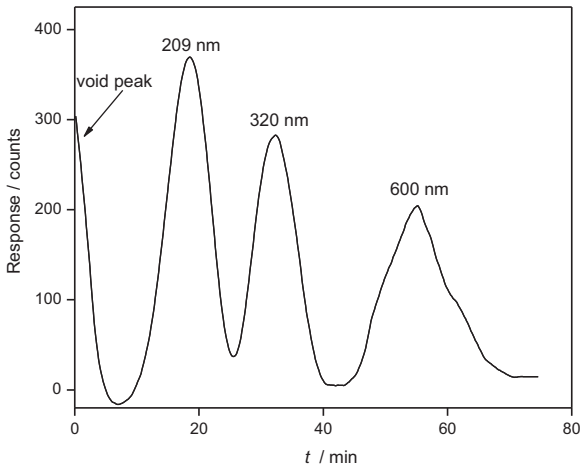

Fig. 1. SdFFF separation of a mixture of 3 PS latex standards (d = 209, 320, 600 nm, respectively) obtained by the field programming method. Injected volume: 20 μl, Solvent: 0.5% (w/v) FL-70 + 0.02% (w/w) NaN_3, flow = 1 ml/min, $\Delta\rho$ = 0.05 g/cm^3, initial rpm = 2500, final rpm = 200, t_1 = 5 min, t_α = −60 min.

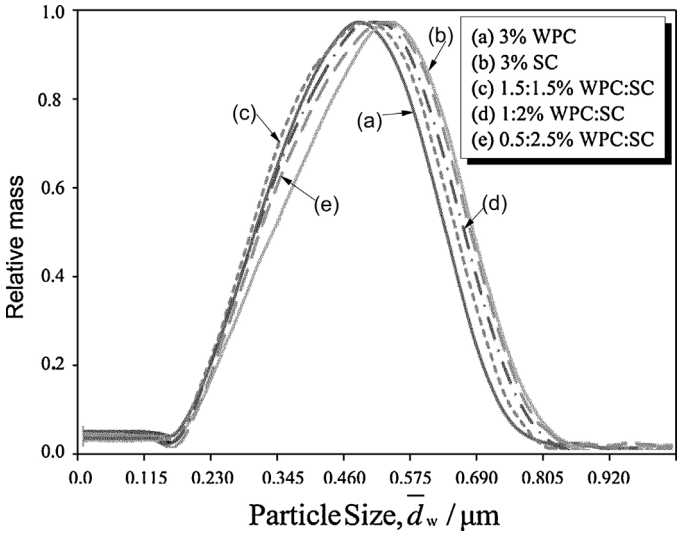

Fig. 2. Droplet size distributions of emulsions made with a binary mixture of SC and WPC at varying weight contributions. Weight average diameter of fat globules is the average of three different emulsions and is deduced from Eq. (4).

corresponding relative masses, w_{r_i}, of the y-axis, we can calculate the weight average diameter, $\bar{d}_w$, of the oil droplets in each fractogram, using an appropriate computer program, by the known relationship for homogeneous spheres:

$$\bar{d}_w = \frac{\sum_i w_i d_{w_i}}{\sum_i w_i} = \frac{\sum_i n_i m_i d_{w_i}}{\sum_i n_i m_i} = \frac{\sum_i n_i \left((1/6)\pi\rho_s d_{w_i}^3\right) d_{w_i}}{\sum_i n_i \left((1/6)\pi\rho_s d_{w_i}^3\right)} = \frac{\sum_i n_i d_{w_i}^4}{\sum_i n_i d_{w_i}^3} \quad (4)$$

where ρ_s is the density of the droplets and m_i is the mass of each spherical droplet. The number of oil droplets n_i at each elution increment dV_{r_i} or dd_{w_i} is supposed to be proportional to the relative mass of class i, w_{r_i} corresponding to this increment as:

$$n_i = \frac{w_i}{m_i} = \frac{w_i/w_{\text{tot}}}{m_i/w_{\text{tot}}} = w_{r_i} \times \frac{w_{\text{tot}}}{m_i} = w_{r_i} \times n_{\text{tot}} \propto w_{r_i} \quad (5)$$

The total number of eluted oil droplets n_{tot} is considered constant.

The correct functioning of the SdFFF system was confirmed by running a PS standard mixture (209, 320 and 600 μm) and comparing the fractograms with that certified by the Postnova Analytics (cf. Fig. 1). Fig. 2 illustrates the particle size distributions of the milk-protein stabilized emulsions as revealed by the fractograms.

The results indicate that all milk protein emulsions exhibited a monomodal size distribution. Whey proteins, for the same protein content, are able to form emulsion droplets with smaller average size diameter compared to sodium caseinate. As the contribution of whey protein fraction increases, the weight average diameter of the fat globule decreases. The average particle size of the emulsion formed solely with sodium caseinate is 0.044 μm higher compared to the corresponding one stabilized with whey protein concentrate, which is a statistically significant difference (Table 2). In the last column of Table 2 (and in the subsequent Table 3) is quoted the standard deviation of the mean value of weight average diameter, as the measurements were carried out in triplicate.

For comparison purposes and in order to show the validity of the SdFFF technique, SEM pictures for the sample containing 3% (w/w) SC were taken (cf. Fig. 3). The weight average diameter found by SEM, from Eq. (4), by measuring the size of about 50 droplets, were 0.533 ± 0.097 μm. This value, taking into consideration the big standard deviation, is in excellent agreement with the value found by SdFFF (0.531 μm). The deviation is about 0.4%, showing that SdFFF is a reliable method for determining the size of the fat globules in the oil-in-water emulsions.

A gradual increase in the particle size of the milk-protein stabilized emulsions is observed for increasing sodium caseinate contribution. The findings of this work, in terms of the particle size distribution of the emulsions formed, are very close to the results presented elsewhere [23]. The average droplet sizes of the emulsions in that case were very close to 0.50 μm, as in this study. However, no differences were reported between the average droplet sizes of the emulsions made with binary mixtures of sodium caseinate and WPC and to those made with sodium caseinate and WPC alone. Sourdet et al. [22] also reported similar particle size distributions for emulsions based on various weight ratios of casein-to-whey proteins, with the values of average median diameter showing a significant increase for casein-free emulsion samples. The results of this study clearly state that whey proteins are better emulsifiers compared to caseins, as the gradual increase in whey protein fraction results in significant decrease in the particle size diameter. This effect might be attributed to partial displacement of caseins from the interface, resulting from competitive adsorption between caseins and whey proteins. It has been stated that

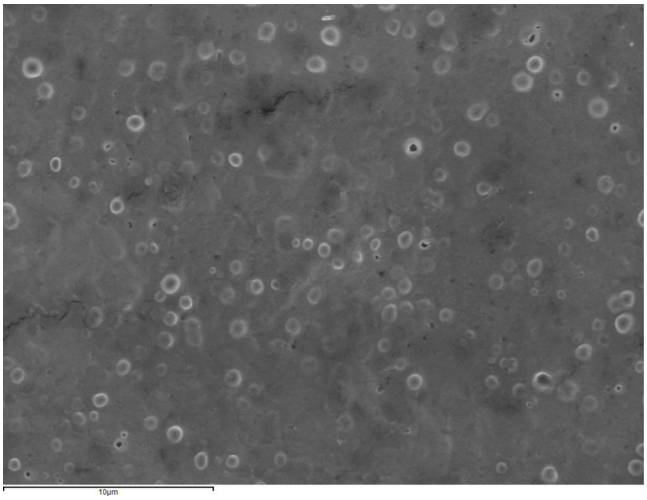

Fig. 3. SEM picture of corn oil-in-water emulsion stabilized by 3.0% (w/w) SC.

Table 2
Comparison of the relative contributions of sodium caseinate (SC) and whey protein concentrate (WPC) in stabilizing the oil-in-water emulsion.

WPC (%, w/w)	SC (%, w/w)	Weight average diameter $\bar{d}_w$ (μm)	Standard deviation[A] (±μm)
3.0	0.0	0.487[a]	0.001
1.5	1.5	0.495[b]	0.001
1.0	2.0	0.510[c]	0.002
0.5	2.5	0.522[d]	0.001
0.0	3.0	0.531[e] (0.533 ± 0.097)[B]	0.001

Means of particle size ($\bar{d}_w$) having different lower case letters (a, b, c, d, e) are significantly different ($P < 0.05$).
[A] The standard deviation of the mean values of $\bar{d}_w$.
[B] The $\bar{d}_w$ found by SEM.

Table 3
Comparison of the relative contributions of Tween (T) and/or SC and/or WPC in stabilizing the oil-in-water emulsions.

T (%, w/w)	WPC (%, w/w)	SC (%, w/w)	Weight average diameter $\bar{d}_w$ (μm)	Standard deviation[A] (±μm)
3.0	0.0	0.0	0.319[a]	0.001
1.0	0.0	2.0	0.338[b]	0.001
1.0	2.0	0.0	0.348[c]	0.002
1.0	1.0	1.0	0.368[d]	0.001

Means of particle size ($\bar{d}_w$) having different lower case letters (a, b, c, d) are significantly different ($P < 0.05$).
[A] The standard deviation of the mean values of $\bar{d}_w$.

the exchange reaction whereby whey proteins can displace caseins from an oil-in-water emulsion is a complicated phenomenon [9]. Nevertheless, other studies indicate that β-lactoglobulin is capable of displacing caseins from an emulsion interface, with the exchange reaction being temperature dependent [29]. In any case, the results of this study indicate that WPC reduces more efficiently the interfacial tension of the freshly made emulsion compared to sodium caseinate, resulting in lower initial droplet-size distributions, which is an important factor affecting the emulsion shelf life [30].

3.2. Effect of surfactant on the average droplet size of oil-in-water emulsions

Tween 80 (polyoxyethylene-20 sorbitan monooleate) was also used (3%, w/w) to form oil-in-water emulsions in the presence or not of milk proteins. As indicated by Fig. 4, all fractograms showed monomodal particle size distributions.

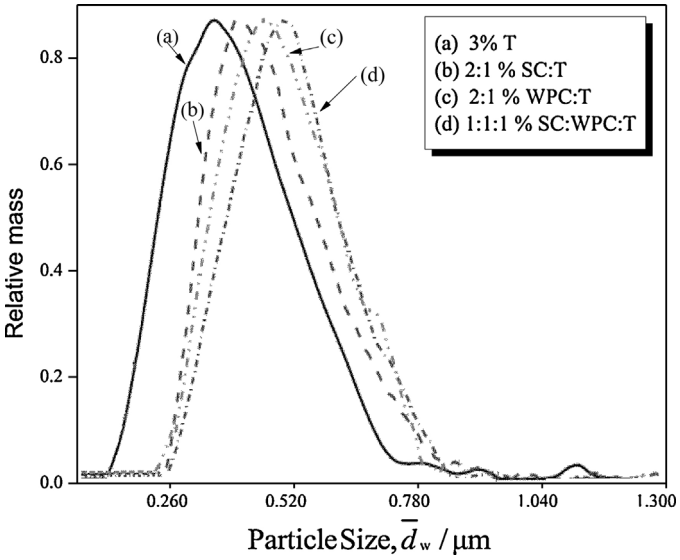

Fig. 4. Droplet size distributions of emulsions made with a mixture of Tween 80 (T) and/or SC and/or WPC at varying weight contributions. Weight average diameter of fat globules is the average of three different emulsions and is deduced from Eq. (4).

Emulsions formed solely with Tween 80 exhibited significantly lower weight average droplet diameter (0.319 μm) compared to samples containing sodium caseinate or WPC (0.531 and 0.487 μm), respectively. According to Dickinson [30], at high surfactant concentrations, low molecular weight molecules such as Tween 80 form a densely packed film around the oil globule and thus, are more efficient in lowering the interfacial tension compared to proteins. The average droplet size of the emulsions increased when sodium caseinate and WPC were added as fractions of the total emulsifier content (Table 2). Interestingly, mixtures of sodium caseinate with Tween 80 (2/1% (w/w), respectively) were more effective emulsifiers compared to the corresponding binary mixtures containing the non-ionic surfactant and WPC, as revealed by SdFFF (Table 3).

It has been documented that the presence of surface-active species such as Tween 80 in an emulsion affects the protein adsorption at the interface [8,31]. It is speculated that small molecule surfactants are capable of adsorbing at vacant holes in the interfacial protein network, resulting in protein bond dissociation with subsequent displacement of the protein molecules [32]. Thus, in the presence of Tween 80, caseins, thanks to their molecular flexibility, are more efficiently rearranged at the interface compared to the globular whey proteins. The results obtained by SdFFF, were further confirmed visually by optical microscopy (cf. Fig. 5).

In this case, it is anticipated that all three different types of emulsifiers are present at the interface in order to form and stabilize the oil droplet. As the concentration of the non-ionic surfactant is considerably low, it would not be possible for Tween 80 to remove significant part of the protein material from the interface because this would lead to insufficient surface coverage of the resulting emulsion [11]. Furthermore, it has been stated that in emulsions formed with both proteins and surfactants, the proteins tend to be the dominant species at the interface, when the surfactant concentration is low [30]. In all mixtures of Tween 80 with sodium caseinate and/or WPC used in this study, the protein fraction contributes to the final emulsion droplet formed for the given surfactant/protein ratios and complete displacement of the milk protein from the interface was not observed. The droplet oil-in-water weight average diameter increases as:

$$3\%\,\mathrm{T} < 1\%\,\mathrm{T} + 2\%\,\mathrm{SC} < 2\%\,\mathrm{WPC} < 1\%\,\mathrm{T} + 1\%\,\mathrm{SC} + 1\%\,\mathrm{WPC}.$$

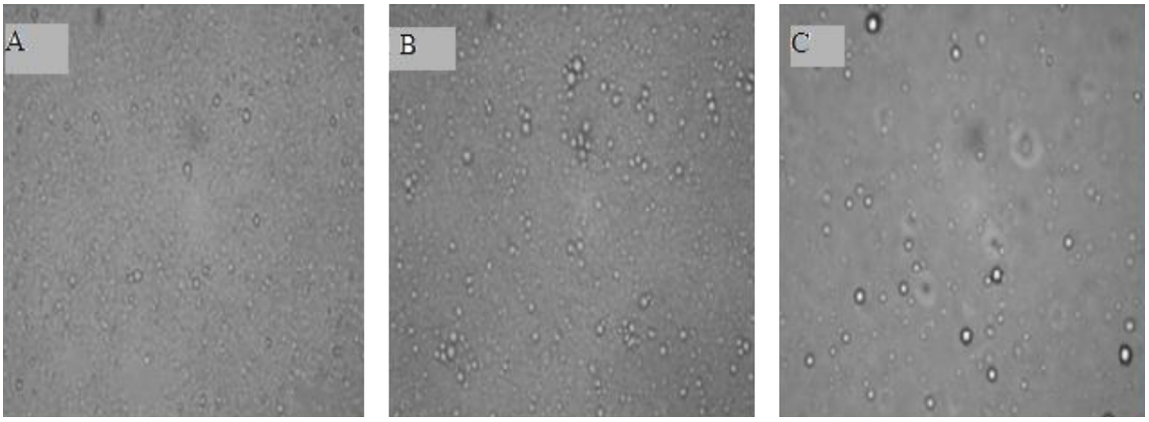

Fig. 5. Micrographs of oil-in-water emulsions stabilized with (A) Tween 80 (3%, w/w), (B) Tween 80:SC (1:2%, w/w) and (C) Tween 80:WPC (1:2%, w/w).

More specifically, in accordance with the findings of Fig. 6, the more effective mixtures of emulsifiers leading to smaller oil-in-water droplets were those containing pure Tween 80, while the less effective mixtures were those containing T, SC and WPC.

3.3. Effect of temperature on the stability of sodium caseinate oil-in-water emulsions

Particle size measurements of heated sodium caseinate emulsions are presented in Table 4.

Emulsions heated at two different temperatures exhibit a similar pattern with respect to particle size distribution. As shown in Fig. 6, a steep and steady increase in the weight average diameter of droplet size is observed for samples subjected to heat treatment for a period of up to 7 h.

At this stage the formation of heat-induced aggregates may be speculated. This temperature-induced effect on the emulsion stability, which is more profound at 80.0 °C, is reversible. Following the initial increase in particle size, the aggregates dissociate and the size distribution drops to values approximating the initial droplet-size. The final particle size distribution (at 70 h) of the emulsion heated at 80.0 °C is even lower compared to the initial droplet size. In this case, heat treatment may have an impact on the structural rearrangement of caseins at the interface, resulting in improved emulsion stability. Similar findings with respect to the effect of heat treatment on the emulsifying ability of sodium caseinate have been reported [33,34]. Caseins, thanks to their structural stability upon heating, are very often used as emulsifiers in oil-in-water emulsions, as thermal processing is common in emulsion product technology [35]. Overall, the findings of this study indicate that caseins are very effective in stabilizing the oil droplet surface, even when heated at relatively high temperatures. Furthermore, several other studies confirm the ability of caseins to withstand severe heat-treatment and to remain unaffected in terms of their emulsifying capacity, thus conferring stability to the oil droplet [10,36]. Depletion flocculation, a common cause of emulsion instability observed at sodium caseinate content above 2% (w/w) [37,38], not concluded in this study. However, further work which involves the formation of emulsions with varying sodium caseinate content and subsequent exposure to the same heat-treatment must be carried out in order to rule out the possibility of depletion flocculation occurring.

Table 4
Weight average diameters, $\bar{d}_{w_i}$, of sodium caseinate emulsions heated at 30.5 and 80.0 °C, at different time intervals, obtained by SdFFF.

30.5 °C		80.0 °C	
Time t_i (h)	Weight average diameter $\bar{d}_{w_i}$ (μm)	Time t_i (h)	Weight average diameter $\bar{d}_{w_i}$ (μm)
0	0.505	0	0.432
2	0.510	2	0.469
4	0.520	7	0.527
6	0.532	9	0.495
7	0.536	12	0.477
9	0.527	48	0.474
11	0.532	70	0.475
14	0.523		
24	0.522		
46	0.511		
70	0.512		

3.4. Rate constants of droplet aggregation of heated sodium caseinate emulsions

The stability of an oil-in-water emulsion is very often related to the ability of this colloidal system to remain structurally unchanged over a certain amount of time [39]. As emulsion stability is regarded as a kinetic phenomenon, the rate constants of the aggregation process upon heating were calculated, in an attempt to quantify the instability process. Pictures taken by the optical microscopy, during the aggregation process, at the temperature 80.0 °C, verified also the variation of the particle size of the oil-in-water emulsions and

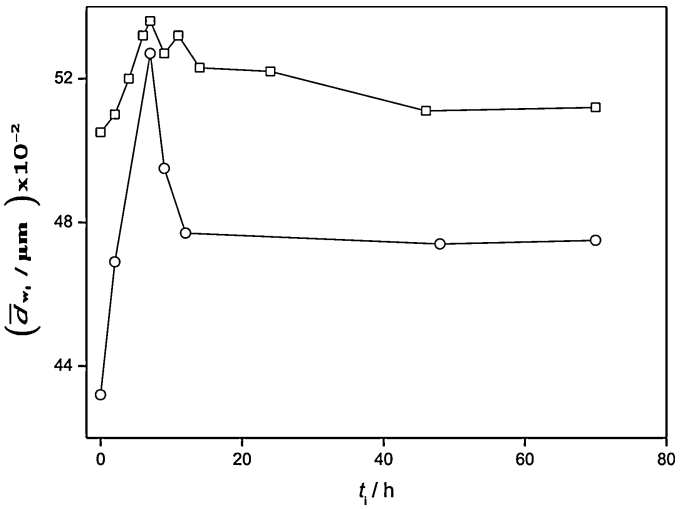

Fig. 6. Variation of the droplet weight average diameter of the oil-in-water emulsion with the time, during thermal processing at 30.5 °C (□) and 80.0 °C (○).

Table 5
Calculated rate constants (k_{app}, k_1, k_{-1} and k_2), stability factors (W) and the total number of effective collisions (ζ) for the droplet aggregation of heated sodium caseinate emulsions.

Temperature	30.5 °C	80.0 °C
Time, t (h)	$0 < t < 7$	$0 < t < 7$
V_0 (cm^3)	6.65×10^{-14}	4.29×10^{-14}
V_{tot} (cm^3)	5.01×10^{-5}	5.09×10^{-5}
N_0 (particles/cm^3)	37.69×10^{9}	59.25×10^{9}
$\bar{d}_{w_0}$(cm)	$5.03 \pm 1.08 \times 10^{-5}$	$4.44 \pm 1.33 \times 10^{-5}$
k_{app} (cm^3 s^{-1})	$2.16 \pm 0.14 \times 10^{-16}$	$5.34 \pm 0.2 \times 10^{-16}$
k_1 (cm^3 s^{-1})	1.42×10^{-11}	3.67×10^{-11}
k_{-1} (s^{-1})	2.13×10^{2}	8.55×10^{2}
k_2 (s^{-1})	3.25×10^{-3}	12.46×10^{-3}
W	6.56×10^{4}	6.86×10^{4}
ζ	1.52×10^{-5}	1.46×10^{-5}

consequently the formation of the aggregates, in agreement with the results obtained by SdFFF.

The rate of oil droplet aggregation at the specified time interval of increasing weight average diameter of droplet size, which depends on the frequency of particle collisions, is described by the expression [40,41]

$$-\frac{dN}{dt} = k_{app}N^2 \tag{6}$$

where N is the number of particles per unit volume of the colloidal suspension in time t and k_{app} is a second order aggregation constant. Given that $N = N_0$ and $t = t_0 = 0$, Eq. (6) may be written as:

$$\frac{1}{N} - \frac{1}{N_0} = k_{app}t \tag{7}$$

and finally as [40]:

$$\bar{d}^3_{w_i} = \bar{d}^3_{w_0} + \bar{d}^3_{w_0}N_0k_{app}t_i \tag{8}$$

where $\bar{d}_{w_i}$ is the weight average aggregate diameter of oil droplets at time t_i, which is calculated from the data points of Fig. 6, at the time interval of increasing average diameter of droplet size and $\bar{d}_{w_0}$ is the weight average diameter of the oil droplets at time $t = 0$. N_0 is the initial number of oil droplets at $t = 0$ which can be calculated from Eq. (9).

$$N_0 = \frac{V_{tot}}{V_0} \tag{9}$$

V_{tot} is the total volume of the corn oil injected in the column, which can be calculated from the known injected volume of the emulsion, the percentage composition of the corn oil (15%, w/w) and the density of the corn oil, supposing that the droplets are consisted only from pure oil, although it is known that the fat globules are coated with a layer of the milk proteins and/or the surfactant. V_0 is the volume of the oil globule formed and is given by the following equation:

$$V_0 = \frac{4}{3}\pi\left(\frac{\bar{d}_{w_0}}{2}\right)^3 \tag{10}$$

Eq. (8) shows that a plot of $\bar{d}^3_{w_i}$ versus t_i should be linear (cf. Fig. 7) with an intercept equal to $\bar{d}^3_{w_0}$ and a slope equal to $\bar{d}^3_{w_0}$ N_0 k_{app}, from which the apparent rate constant (k_{app}) for aggregation can be calculated (Table 5).

In order to show if the oil droplet aggregation is a diffusion-controlled rapid aggregation in the absence of any energy barrier or a slow aggregation process in the presence of an energy barrier, and thus, to calculate the oil droplet stability factor, the

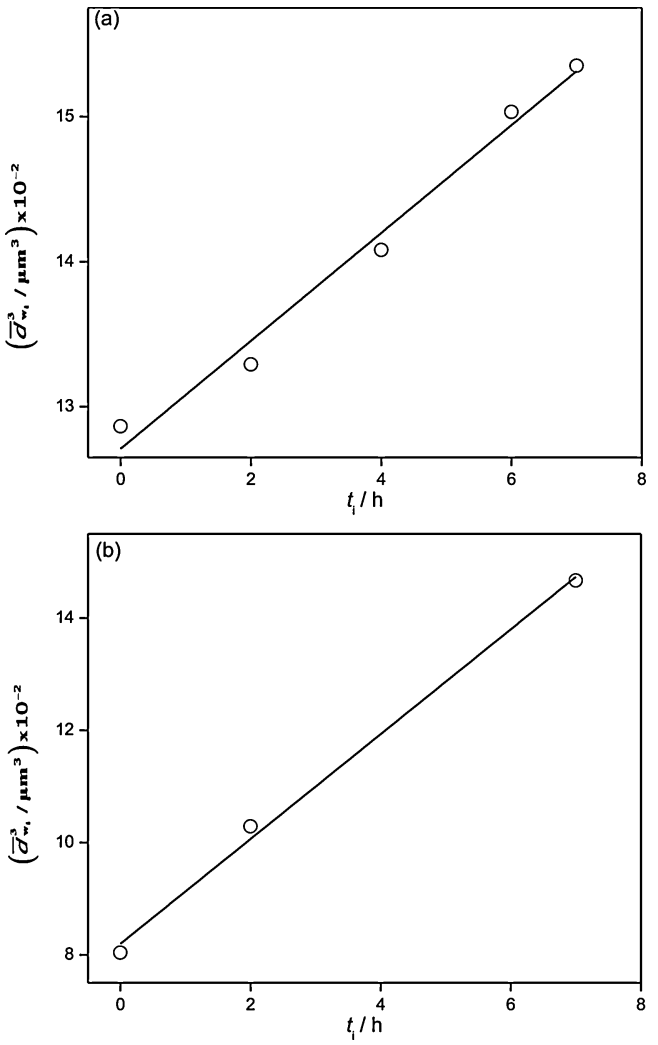

Fig. 7. Plot of $\bar{d}^3_{w_i}$ versus t_i for the aggregation of sodium caseinate oil-in-water emulsions during thermal processing at 30.5 °C (a) and 80.0 °C (b). The solid line represents linear regression fit.

respective rate constants k_r and k_s are calculated from the known van Smoluchowski equations [41,42]:

$$u_r = -k_rN_0^2 \tag{11}$$

$$u_s = -k_sN_0^2 \tag{12}$$

where u_r and u_s are the rates for the rapid and slow aggregation processes, respectively.

The rate constant for the bimolecular rapid aggregation of the oil droplets, k_1, can also be determined from the following equation [43]:

$$k_1 = \frac{8kT}{3n} \tag{13}$$

where n is the viscosity of the medium. For water, we calculate from Eq. (13) a k_1 value of 1.42×10^{-11} cm^3 s^{-1} at 30.5 °C and of 3.67×10^{-11} cm^3 s^{-1} at 80.0 °C. These values are about five orders of magnitude higher than the values of k_{app} actually measured at the same temperatures. This means, that aggregation rates are slower than those expected if the process was simply diffusion-controlled. In this case, extra repulsive hydration forces [44] occurring at close approach of oil droplets, may be involved in the observed slow processes. Since the calculated value for k_{app} describing the overall process is lower than the calculated k_1 values, there must be rapid

equilibration of the individual oil droplets and their intermediate complexes followed by a slower step of irreversible aggregation. Thus, the proposed mechanism for the aggregation process of the oil droplets would be:

$$\text{Individual oil droplets} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \text{Intermediate complex of oil droplets} \xrightarrow{k_2} \text{Aggregates of oil droplets} \quad (14)$$

where k_1 and k_{-1} are the rate constants for the formation and the dissociation of the intermediate complex, respectively, while k_2 is the rate constant representing the rate determining step in the aggregation process. This general mechanistic scheme explains the forms of the plots giving the variation of $\bar{d}_{w_i}$ versus t_i (cf. Fig. 6). The ascending part of the two curves, when $0 < t < 7$ h, can be attributed to the droplet aggregation, which happens when $k_1 >> k_{-1}$. On the other hand the descending part of the curves, when $7 < t < 70$ h, can be attributed to the disaggregation process of the oil-in-waters aggregates, which happens when $k_1 << k_{-1}$. According to the mechanism (14) the k_{app} is given by the relation:

$$k_{app} = \left(\frac{k_1}{k_{-1}}\right) k_2 \quad (15)$$

Straightforward calculation of k_1 from Eq. (13) and of k_{-1} from the following relation [43]

$$k_{-1} = \frac{16kT}{\pi n \bar{d}_{w_0}^3} \quad (16)$$

allows the determination of the k_2 from Eq. (15), as the k_{app} values have been already calculated from the slopes of the plots of Fig. 7 (Table 5).

Finally, the stability factor ($W = k_r/k_s = k_1/k_{app}$), as well as the fraction of the total number of collisions, which are effective in producing stable aggregates ($\zeta = k_2/(k_{-1} + k_2)$), can be calculated (Table 5). The W values are relatively high indicating that the oil droplets are very stable, even at the higher temperature of 80.0 °C, while the ζ values are considerably low indicating that the fraction of the total number of collisions which are effective in producing a stable oil droplet aggregate is very low (an order of magnitude of 10^{-5}).

The results of this study indicate that the rate of oil droplet aggregation, as it is expressed by the rate constant k_2 is nearly 4 times higher when emulsions are heated at 80.0 °C compared to thermal processing at 30.5 °C. Thus, non-surprisingly the process of particle aggregation is faster at higher temperatures. That is because in that case the high thermal energy of the particles is used to overcome the repulsive forces between them. On the other hand, for both emulsions the aggregation process takes place for the same period of thermal processing, which is 7 h. As a result, the size of the aggregated oil droplets is larger for the samples heated at 80.0 °C, for the same processing time. Conclusively, the duration of thermal processing is important in terms of the onset and completion of the aggregation process, whereas the degree of heating determines the size of the aggregated particles formed. The fact that the rate constant for the oil droplet aggregation at 80 °C is approximately 4 times higher than that at 30.5 °C confirms the existence of the aggregation process, which was also verified by optical microscopy.

4. Advantages of SdFFF

In contrast to traditional particle sizing techniques which work in the batch mode, the SdFFF technique physically separates each particle fraction prior to sizing. This avoids numerous disadvantages of the batch techniques such as, low size resolution, under estimation of smaller by larger particles and discrimination. In SdFFF no special sample treatment is necessary, as they can be injected directly, allowing the separation and characterization of quite complex samples.

Comparing SdFFF with other advanced techniques for the measurement of the particle size, such as dynamic light scattering (DLS) technique, the SdFFF has higher resolution, higher size range for analysis, as well as higher quantitation. Its detection sensitivity is lower compared to that of DLS.

The precision of the SdFFF technique in measuring particle size of the oil-in-water emulsions, and consequently in studying the kinetics of the droplet aggregation, was calculated from the data of Table 2 by using the formula:

$$\text{Precision}\,(\%) = 100 - 100 \times \frac{\text{deviation}}{\bar{d}_w} \quad (17)$$

From the values quoted a precision varying from 99.61% to 99.81%, with a mean value of 99.76%, was calculated, showing that the SdFFF technique is a very precise method for measuring particle size distributions for sodium caseinate emulsions.

Comparison of the weight average diameter of the droplets determined by SdFFF with that found by SEM pictures shows a deviation of about 0.4% indicating the validity of the SdFFF, which is a separation method, in studying the kinetics of aggregation for the oil-in-water emulsions in the presence of milk proteins and/or surfactants.

5. Conclusions

Understanding milk protein functionality at interfaces is essential in order to develop emulsion-based food products. SdFFF was employed to monitor changes in the particle size distribution of emulsions stabilized with varying proportional contributions of sodium caseinate, WPC and Tween 80. Emulsifying ability follows the order Tween 80 > WPC > sodium caseinate. The results of this study may be explained in terms of the structural differences between the three types of emulsifiers. The formation of a dense layer surrounding the oil droplet which reduces the interfacial tension is a "task" carried out better by the efficient packing of low molecular weight surfactants or compact protein molecules. On the other hand, emulsions containing sodium caseinate as the sole emulsifier were heat-stable, even at relatively high temperatures (80.0 °C). Elevated temperatures during thermal processing may have a beneficial effect on the structural rearrangement of caseins at the interface. Sodium caseinate emulsions exhibited an increase in particle size distribution caused by heat-induced droplet aggregation, followed by a decrease to approximately the initial droplet size. The process of droplet aggregation is temperature dependent, as higher temperature increases significantly the rate of particle aggregation. The rate constant of droplet aggregation for sodium caseinate emulsion heated at 80.0 °C is approximately 4-fold higher compared to one heated at 30.5 °C. The formation of an intermediate complex of oil droplets followed by the formation of stable aggregates, as revealed by the kinetics of the aggregation process, is proposed for the first time in this work. Based on this mechanistic scheme, which was also presented previously [40,45] for the aggregation of hydroxyapatite particles, the following physicochemical quantities which are very important in explaining the stability of the oil-in-water emulsions, were determined:

(i) apparent rate constants for the slow aggregation of the oil-in-water emulsions,
(ii) rate constants for the formation of the intermediate complex,
(iii) rate constants for the dissociation of the intermediate complex,

(iv) rate constants for the formation of the aggregates from the intermediate complexes (the rate determining step),
(v) stability factors for the aggregates, and
(vi) the total numbers of effective collisions in producing the stable aggregates.

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